CLINICAL CHARACTERISTICS OF PATIENTS WITH POSITIVE CARBAPENEM RESISTANT *ENTEROBACTERIACEAE* (CRE) AND ITS GENOTYPE

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ABREVIATIONS

ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
CAP	Community Acquired Pneumonia
CBD	Continuous Bladder Drainage
CAUTI	Catheter Associated Urinary Tract Infection
CRE	Carbapenem Resistant *Enterobacteriaceae*
CP-CRE	Carbapenemase Producer - Carbapenem Resistant *Enterobacteriaceae*
CDC	Centres for Disease Control
CLABSI	Central Line Associated Bloodstream Infection
CLSI	Clinical and Laboratory Standards Institute
CRBSI	Catheter Related Bloodstream Infection
CRKP	Carbapenem Resistant *Klebsiella pneumoniae*
DEPC	Diethylpyrocarbonate
DHP - I	DeHydroPeptidase I
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetra Acetic Acid
ESBL	Extended Spectrum of Beta-Lactamases
ETT	Endotracheal secretion
GES	Guaiana-Extended Spectrum
GIM	German-imipinemase
HAI	Health care associated infection
HAP	Hospital Acquired pneumonia

HRPZ II	Hospital Raja Perempuan Zainab II
HUSM	Hospital Universiti Sains Malaysia
ICU	Intensive Care Unit
IMI	Imipinem-Hydrolyzing
IMP	Imipinemase Metallo-beta-lactamase
KPC	*Klebsiella Pneumoniae* Carbapenemase
LPS	Lipopolysaccharide
MBL	Metallo-Beta-Lactamases
MIC	Minimum Inhibitory Concentration
MHA	Muller-Hinton Agar
MHT	Modified Hodge Test
MR	Methyl Red
NDM-1	New Delhi Metallo-beta-lactamase - 1
NICU	Neonatal Intensive Care Unit
NMC	Non-Metalloenzyme Carbapenemase
OXA	Oxacilinase
PAMP	Pathogen - Associated Molecular Pattern
PBP	Penicillin-Binding Proteins
PCR	Polymerase Chain Reaction
SAPSS	Software Package used for Statistical Analysis
SIM	Sulphate Indole Motility
SIM-1	Seoul-Imipinemase
SME	Serratia Marcescens Enzyme
SPM-1	Sao Paolo Metallo-beta-lactamase
SSI	Surgical Site Infection

TBE	Tris/Borate/EDTA
TNF-α	Tumor Necrosis Factor-alpha
TSI	Triple Sugar Iron
UTI	Urinary Tract Infection
UV	Ultra Violet
VAP	Ventilator Associated Pneumonia
VIM	Verona Integron-encoded Metallo-beta-lactamase

ABSTRAK

PERILAKU KLINIKAL PESAKIT DENGAN POSITIF

CARBAPENEM-RESISTANT *ENTEROBACTERIACEAE* (CRE) DAN GENOTIP

Pengenalan

Carbapenem-resistant *Enterobacteriaceae* (CRE) adalah mikroorganisma yang sedang muncul dan berkembang di seluruh dunia dan bakal menimbulkan ancaman kepada tatacara penjagaan kesihatan. Gen yang boleh dipindah milik telah menyebabkan kerintangan kepada banyak kumpulan antibiotik yang membawa kepada kesukaran rawatan dan kadar kematian yang tinggi. CRE adalah biasanya berkaitan dengan penjagaan kesihatan.

Jangkitan invasif dengan CRE telah dikaitkan dengan kes kematian yang tinggi. Mengenal pasti pesakit dengan CRE positif adalah penting untuk menghalang penyebaran organisma berdaya tahan ini yang berpotensi untuk menyebabkan jangkitan. Oleh yang demikian, kajian ini dijalankan untuk mengetahui signifikasi dan gen bagi CRE berserta perilaku klinikal setiap pesakit yang positif CRE di HUSM.

Tatacara kajian

Kajian ini dijalankan di Hospital Universiti Sains Malaysia bermula Ogos 2013 sehingga Disember 2015. CRE positif telah dikenal pasti daripada Makmal Mikrobiologi Perubatan & Parasitologi. CRE yang telah di kenalpasti kemudian diuji untuk gen *bla*-$_{\text{NDM.}}$ Semua yang negatif *bla*-$_{\text{NDM}}$ kemudiannya akan diuji untuk gen *bla*-$_{\text{IMP}}$ menggunakan teknik PCR. Rekod pesakit di semak dan kemudiannya data yang dikumpulkan dianalisa menggunakan perisian SPSS Statistik versi 22. Analisa secara deskriptif dijalankan bagi kedua-dua kategori dan

pemboleh ubah bebas berangka dan seterusnya diukur menggunakan frekuensi, peratusan dan median interquatile range (IQR) secara respektif. Bagi kategori analisa data, *Fisher exact test* and *Pearson Chi-square test* digunakan.

Keputusan

Antara 136 CRE sampel daripada 114 pesakit, 60 (44.1%) adalah daripada sapuan rektum diikuti oleh urin [25 (18.4%)], cecair badan [19 (14.0%)], darah [15 (11.0%)], kahak [9 (6.6%)] dan sapuan /tisu [8 (5.9%)]. Organisma yang paling banyak adalah *Klebsiella pneumoniae* (n= 126, 92.7%), diikuti oleh *Enterobacter aerogenes* (n=4, 2.9%), *Enterobacter cloacae* dan *Klebsiella ozanae* [dua (1.5%)]. Satu (0.7%) sampel adalah Ci*trobacter freundii* dan *Escherichia coli*. Genotip yang paling banyak adalah *bla*-$_{NDM1}$ (n=112, 82.4%) dan hanya satu (0.7%) *bla*-$_{IMP}$. Kebanyakan daripada pesakit, 81(71.1%) telah dikoloni oleh CRE, 23 (20.2%) telah dijangkiti dan sepuluh (8.8%) pesakit adalah telah dikoloni dan kemudiannya dijangkiti. CRE kebanyakannya terjadi di kalangan pesakit yang sudah mempunyai penyakit lain sebelum ini (n=89, 78.1%). Darah tinggi (53.5%), kencing manis (43.9%) dan penyakit buah pinggang kronik (36.0%) adalah merupakan penyakit yang paling banyak ditemui. Kebanyakan pesakit mempunyai kemasukan ke hospital secara berpanjangan (n=90, 78.9%), kemasukan ke ICU (n=69, 60.5%) dan sejarah kemasukan ke hospital (n=70, 61.4%) semasa CRE di kesan. Jangkitan yang kerapkali berlaku adalah jangkitan saluran darah (n=14, 35.0%) dan pneumonia (n=14, 35.0%). CRE-Jangkitan saluran darah (p=0.002) dan CRE-pneumonia ($p<0.001$) adalah sangat berkait dengan kematian.

Kesimpulan

Hasil daripada kajian yang telah dijalankan, adalah didapati kebanyakan daripada sampel CRE adalah *Klebsiella pneumoniae* dan kebanyakannya daripada pesakit yang telah di koloni CRE. Gen *bla*-$_{NDM}$ adalah gen yang paling banyak dikesan dalam sampel CRE. Lebih 50% daripada pesakit yang positif CRE adalah terdiri daripada mereka yang telah dimasukkan ke hospital secara berpanjangan, kemasukan ke ICU dan pernah dimasukkan ke hospital. Sebagai tambahan, CRE-jangkitan saluran darah and CRE-pneumonia adalah jangkitan yang paling banyak ditemui dan kebiasaannya mengakibatkan kematian.

CHAPTER 1

INTRODUCTION

1.1 Overview of Carbapenem Resistant *Enterobacteriaceae* (CRE)

Carbapenem Resistant *Enterobacteriaceae* (CRE) has emerged globally and effective infection control measures are important to prevent further spread. These *Enterobacteriaceae* which normally resides in human gastrointestinal tract are now become more virulence and causing harmful diseases to human mainly those staying in hospital and possibility in community as well. Early detection of these organism and early recognition of the patients at risk of getting this 'super bug' is necessary. CRE is associated with higher mortality (40%) compared to carbapenem sensitive *Enterobactericeae* (Hussein *et al.*, 2013).

Carbapenem that previously work as a miracle drug for Gram negative organisms are no longer able to fight them and leading to the use of old drugs such as Polymyxin which was first found in 1947 (Storm *et al*., 1947). It was abandoned and not actively used for years due to its neurotoxic and nephrotoxic effect (Evans *et al*., 1999).

There are few complex mechanisms of resistance in CRE which include production of carbapenemase enzymes labelled as Carbapenemase Producer - Carbapenem Resistant *Enterobacteriaceae* (CP-CRE). The resistant genes are located in the plasmid that easily transferable from one species to another. CP-CRE is important to be recognized as it will determine the choice of therapeutic management and extent of infection control measures that need to be implemented.

Metallo-beta-lactamase enzymes are the most predominant cause of CRE in Malaysia which causes resistant not only to carbapenem but to other beta-lactam drug. Detection of these

enzymes possess difficulty in the laboratory as the current use of phenotypic detection of *carbapenemases* which is the Modified Hodge test has lower sensitivity to detect Metallo-beta lactamase compared to other enzymes such as serine beta-lactamase (eg: KPC gene) which has better detection.

Molecular methods in detecting gene responsible for CRE are now better option for *carbapenemase* gene detection particularly in the reference laboratory. It provides accurate, reliable and fast results which is necessary for treatment and infection control measures.

1.2 Rationale of the Study

CRE is one of the national surveillance organisms under Infection Control Unit, Medical Development Division in Ministry Of Health, Malaysia. Based on the national surveillance data in 2015, incidence for CRE was increasing from 0.02 per 100 admissions in 2013 to 0.05 per 100 admissions in 2015. Although the rate was still low compared to other national surveillance organisms, the devastating effect and the possible of rapid spread of these organisms warrant continuous attention to these organisms. Furthermore reported prevalence of CRE was increasing worldwide. As in US, CDC in March 2013 reported that 3.9% of short stay acute care hospital and 17.8% of long term acute care hospitals reported at least one CRE health care associated infection.

Infection prevention control strategies and attention to colonization pressure will be key factors in minimizing the spread of CRE infection or colonization (G.Bushnell *et al*., 2013). Patients colonized with CRE are thought to be a source of transmission in healthcare settings. Based on CDC guidelines 2012, recognizing CRE colonized or infected patients and place on contact precautions are one of the prevention strategies for CRE. Therefore we want to determine the clinical characteristics of patient with CRE in our settings as well as the burden of CRE infection and colonization in our settings. By knowing this, infection control

measures can be taken earlier as screening among patient who had higher risk can be done earlier.

To date, NDM-1 gene was the commonest gene detected in CRE cases in Malaysia. Based on previous report done in HUSM, besides NDM-1 gene, *bla*-$_{IMP4}$ gene was also detected in 1.87% of the cases (Hamzan *et al.*, 2015). However that study only involved small number of confirmed CRE cases (13 cases) and our study was done with a bigger number of sample size. The proportion of *bla*-$_{NDM-1}$ gene and *bla*-$_{IMP4}$ also can be determined in our settings in order to have a better representative distribution of the population.

Based on this study, we were able to determine the type of infection that contributes to higher mortality among CRE patients. CRE-bloodstream infections were associated with high mortality rate (41.6%) (Tumbarello *et al.*, 2012). Therefore it is to alert the management team regarding the higher risk of mortality in certain type of infection where CRE is isolated and prompt a judicious antibiotic usage including antimicrobial combining regime.

CHAPTER 2

LITERATURE REVIEW

2.1 *Enterobacteriaceae*

Enterobacteriaceae are Gram negative bacilli that are non-spore forming, facultative anaerobes that ferment glucose and other sugars, reduce nitrate to nitrite, produce catalase and do not produce oxidase except for *Plesiomonas*. Most are motile via peritrichous flagella except for *Klebsiella* spp and *Shigella* spp. These organism are the most commonly encountered organism in the laboratory from the clinical samples and mostly pathogenic (Farmer, 1995). Listed below are the hierarchy taxonomy of *Enterobacteriaceae*.

Domain : Bacteria

Phylum : Proteobacteria

Class : Gammaproteobacteria

Order : Enterobacteriales

Family : *Enterobacteriaceae*

There are 53 genera and over 170 named species of bacterial family *Enterobacteriaceae* and in which 26 of them are medically important and known to cause human infections. The genera keep on changing with the use of DNA sequencing on further identification of the genera and species (Nhung *et al.*, 2007). Members of family *Enterobacteriaceae* are *Arsenophonus, Biostraticola, Brenneria, Buchnera, Budvicia, Buttiauxella, Calymmatobacterium, Cedecea, Citrobacter, Cosenzaea, Cronobacter, Dickeya, Edwardsiella, Enterobacter, Erwinia, Escherichia, Ewingella, Gibbsiella, Hafnia, Klebsiella,*

Kluyvera, Leclercia, Leminorella, Levinea, Lonsdalea, Mangrovibacter, Moellerella, Morganella, Obesumbacterium, Pantoea, Pectobacterium, Phaseolibacter, Photorhabdus, Plesiomonas, Pragia, Proteus, Providencia, Rahnella, Raoultella, Saccharobacter, Salmonella, Samsonia, Serratia, Shigella, Shimwellia, Sodalis, Tatumella, Thorsellia, Trabulsiella, Wigglesworthia, Xenorhabdus, *Yersinia* and *Yokenella*. Out of this, the medically important genera are *Citrobacter, Edwarsiella, Enterobacter, Escherichia, Hafnia, Klebsiella, Morganella, Pantoea, Plesiomonas, Proteus, Providencia, Salmonella, Shigella, Serratia* and *Yersinia.*

2.2 Epidemiology of *Enterobacteriaceae*

Enterobacteriaceae are widely distributed in the environment such as in soil and water. Besides that members of *Enterobacteriaceae* were also predominantly found in the lower gastrointestinal tract of various animals. *Enterobacteriaceae* were also found to be colonizing at oropharyngeal airways particularly in patients who had artificial ventilation either by endotracheal intubation or tracheostomy. More than 50% of similar microorganism were detected in lower respiratory tract sample, leading to 30% of the cases become infection (Van Uffelen *et al.*, 1984). Members of *Enterobacteriaceae* causes wide variety of infections include hospital and community setting. They can affect both inpatient who had pre-existing illness or healthy people due to their virulence. They can be isolated from any clinical samples for examples urine, blood, sterile body fluids and specimen from respiratory tract.

2.3 Structure of *Enterobacteriaceae*

Generally members of *Enterobacteriaceae* are rod shaped organisms, 1 to 3µm in length and 0.5µm in diameter. Genomic construction comprises of single circular chromosomes and may include multiple plasmids of various sizes in the cytoplasm. It has both inner and outer membrane whereby in between were periplasmic space that contains peptidoglycan cell layer. The inner membrane is impervious to polar molecules, regulates the passage of nutrients, metabolites, macromolecules and information in and out of the cytoplasm and maintain the proton required for energy storage. Periplasmic space is an aqueous environment with high concentration of proteins and peptidoglycans. All types of proteins involve in biogenesis of peptidoglycans, capsules and lipopolysaccharide are available in this space. Peptidoglycan cell layer in Gram negative bacteria is only a thin layer as opposed to Gram positive bacteria who had a thick layer of peptidoglycan. This peptidoglycan is composed of alternating N-acetylglucosamine and N-acetylmuramic acid amino sugars joined with short peptide. It is responsible for the shape and osmotic stability of the Gram negative organism.

The outer membrane of Gram negative bacteria is composed of asymmetrical lipid bilayer. The inner leaflet is composed of phospholipid where else in the outer leaflet mainly made of lipopolysaccharide (LPS). Porin proteins are present through the outer membrane as a passage for hydrophilic molecules. Lipopolysaccharides consist of Lipid A, core oligosaccharides and O antigen (Raetz and Whitfield, 2002). The O antigen is the basis for serogroup classifications. Flagella are flexible surface appandages that in charge to rotate and drive forward the bacteria in the liquid environment. H- antigen types that is present on the flagellin is responsible for one of the serotyping. Most *Enterobacteriaceae* had pili that extend from the surface and are thinner than flagella. Pili is important in adhesions to host cells, in autoaggregation and genetic exchange through conjugations. Besides that

Enterobacteriaceae also possess capsule that both link to LPS or α-glycerol phosphate and form the basis of K-antigen serotyping.

2.4 Pathogenesis of *Enterobacteriaceae* causing infections

Lipid A, also known as endotoxin which serves as the backbone of the LPS, is one of the pathogen-associated molecular patterns (PAMP) that can be recognized by pattern-recognition receptors such as Toll-like receptor 4. It triggers the innate immune response in host cells. Other PAMP that can trigger the innate immune response would be flaggelin (a protein that is present in bacteria flagella) and unmethylated CpG dinucleotides that is numerous in bacterial genomic DNA, both were present in Gram positive and Gram negative bacteria (Matsuura, 2013). However, LPS is only found in Gram negative bacteria. These Toll-like receptors are express on the monocytes and macrophages. It will activates phagocytic activity and release of pro-inflammatory cytokines such as Tumor necrosis factor-alpha (TNF-α), Interleukin-6 and other high inflammatory cytokines to kill the pathogens. However, excessive and prolonged uncontrolled activation of immune systems, particularly excessive TNF-α causes pathological effects characterized by endotoxic shock (Rosenfeld and Shai, 2006).

2.5 Infection with *Enterobacteriaceae*

Enterobacteriaceae can cause wide variety of infections include healthcare associated infections and community acquired infections. There are few types of healthcare associated infections which include central line associated blood stream infection, ventilator associated pneumonia, catheter associated urinary tract infection and surgical site infection which will be discussed further below. *Escherichia coli* 0157: H7 is well known to cause hemorrhagic colitis (90% of cases) and haemolytic uremic syndrome (10% of cases) in human (Tarr, 1995). Besides that *Escherichia coli* were also known to cause urinary tract infection which is the commonest cause of extra intestinal infection due to the presence of α-hemolysins, P-fimbriae and also Shiga toxins (Johnson and Russo, 2002; Toval *et al*., 2014). *Proteus mirabilis* and *Providencia stuartii* is the leading cause of catheter associated urinary tract infection and merely due to ability to form urease- dependant manner which co-infection can further promote urolithiasis and bacteraemia (Armbruster *et al.*, 2014). The organism also able to form biofilm that promote colonization on the catheter (Jacobsen and Shirtliff, 2011). *Klebsiella* species is found higher in rectal carriage of patients being hospitalized rather than not hospitalized (Montgomerie, 1979). It is mainly due to usage of antibiotics that alter normal bowel flora rather than admission to hospital or immunosuppressive therapy (Montgomerie *et al.*, 1970). Besides colonization at the nose and pharynx which can lead to lower respiratory tract infection, *Klebsiella* species also can present on skin of normal healthy subject which later on can cause skin and soft tissue infections in the presence of wound (Marples, 1965). Bacteraemia and intrabdominal infections are other clinical manifestation of *Enterobacteriaceae*.

2.5.1 Health Care Associated Infection (HAI)

Based on Centers for Disease Control (CDC) guideline 2016, HAI was defined as an infection where the date of event of site specific infection criterion occurs on or after the 3rd calendar day of admission to an inpatient location where day of admission is consider as day one. HAI can be further divided as central line associated bloodstream infection, surgical site infection, ventilator associated pneumonia and catheter associated urinary tract infection.

Bloodstream infection is defined by the presence of pathogenic organism in the blood culture that is regard as infection, not contamination (Shah *et al.*, 2013). It can be further divided into primary bloodstream infection or secondary bloodstream infection. Primary bloodstream infection is laboratory confirmed bloodstream infection and whereby the recovery of the pathogen is not secondary to an infection at other sites (CDC, 2016). Secondary bloodstream infection is defined when there is matching organism between blood culture and site specific infection or the positive blood culture is occurring within the infection period for the site specific infections (CDC, 2016).

Central line associated bloodstream infection (CLABSI) is laboratory confirmed blood stream infection where by central line or umbilical catheter is presence for more than 2 days on the date of event, with day one is consider when the day of device placement took place and the line was also in place on the infectious date or the day before (CDC, 2016). CLABSI has different meaning and purpose compare to catheter related bloodstream infection (CRBSI). A CRBSI is regard as clinical definition, used when diagnosing and treating patients, which needs specific laboratory testing that can identifies the catheter as the source of the bloodstream infections (CDC, 2011). CRBSI is used for research investigations of the risk factors and pathogenesis of vascular catheter related bloodstream infections where else CLABSI is used for surveillance purposes and not related to research (Shah *et al.*, 2013).

Catheter associated urinary tract infection (CAUTI) is defined as presence of UTI where an indwelling urinary catheter was in position for more than 2 days and that indwelling urinary catheter was in place on the date of event or the day before (CDC, 2016). Indwelling catheter is a drainage tube (Foley catheters) that is introduced into the urinary bladder through the urethra, is left in place, and connected to a drainage bag. Condom, in-and-out catheters and nephrostomy tubes are not considered as indwelling catheter. Suprapubic catheters is not indwelling catheter too unless a Foley catheter is also present (CDC, 2016). Symptomatic urinary tract infection is defined as presence of positive urine culture with no more than 2 organisms and at least one of the bacteria should have more than 10^5 colony forming unit per ml and there is presence at least one of the symptoms either fever (more than 38°C), frequency, urgency, dysuria, costovertebral angle pain and suprapubic tenderness in which there is no other recognized cause present (CDC, 2016). Prolonged catheterization and female due to short urethra were commonly seen as risk factors for developing CAUTI (Tambyah, 2004).

Pneumonia is defined based on collaborative of clinical, imaging and laboratory criteria. Pneumonia is presence of new lung infiltrate plus clinical evidence that the infiltrate is of an infectious origin, which include new onset of fever, purulent sputum, leukocytosis, and reduce in oxygenation (Mandell *et al.*, 2007). It can be further divided into community acquired pneumonia (CAP), hospital acquired pneumonia (HAP) and ventilator associated pneumonia (VAP). For HAP, it is defined whereby pneumonia that occur after 48 hours of admission and not incubating at the time of admissions. VAP is defined as pneumonia that occur after 48 hours of endotracheal intubation (Kalil *et al.*, 2016). Mortality rates for VAP was 38% (Tejerina *et al.*, 2006). VAP was associated with prolonged ICU stay, hospital stay, and mechanical ventilation(Muscedere *et al.*, 2010).

Surgical site infection (SSI) is defined as infections that occur up to 30 days of surgery or up to one year in patients receiving implant (Owens and Stoessel, 2008). Patient also should have at least one of these criteria which are; purulent discharge from incisional site, organisms able to be identified from aseptic specimen taken, or when culture is not performed patient had sign and symptoms of inflammation or diagnosis of SSI were made by surgeon or attending physicians (CDC, 2016). In US, SSI rate was 1.9% in 2006-2008 and mortality rate for SSI was 3% (CDC, 2016). The risk factors would be advanced age, underlying comorbidity, longer duration of surgery and complexity of surgery (Korol *et al.*, 2013). The most common site of surgery SSI being identified was colon surgery (32.3%) and most common organisms were *Escherichia coli* (20.4%), *Pseudomonas aeruginosa* (19.3%) and *Candida albicans* (13.7%) (Ballus *et al.*, 2015).

2.6 Treatment for *Enterobacteriaceae*

Generally *Enterobacteriaceae* are sensitive to β-lactam drugs such as cephalosporin group, especially the third generation of cephalosporin such as cefotaxime, ceftriaxone and ceftazidime that have more stable activity for β-lactamases. However, TEM-1 and SHV-gene were commonly present in *Enterobacteriaceae* and confer resistance to ampicillin, amoxicillin, other penicillin group and earlier generation of cephalosporin but were still sensitive to third generation cephalosporins and monobactam. Nowadays the gene become mutated and produce Extended spectrum of beta-lactamases (ESBL) that are able to hydrolyse even third generation cephalosporin and monobactam (Paterson, 2006). There are other resistance genes in ESBL such as CTX-M and OXA-type ESBLs. For CTX-M type ESBL, higher MIC was seen with cefotaxime than ceftazidime (Walther-Rasmussen and Høiby, 2004). OXA-type ESBL more commonly seen in *Acinetobacter* species and

Pseudomonas aeruginosa and rarely in *Enterobacteriaceae* (Bonomo and Szabo, 2006). Carbapenem is the treatment of choice for ESBL bacteraemia (Vardakas *et al.*, 2012). Previous usage of carbapenem is one of the risk factors for CRE (Hussein *et al.*, 2009a)

2.7 Carbapenem

The backbone of carbapenem is thienamycin which is from *Streptomyces cattleya*. Thienamycin has high affinity to penicillin binding protein and stable against β-lactamases. However this thienamycin is not stable in aqueous environment and in mild base hydrolysis (Papp-Wallace *et al.*, 2011). Carbapenem is a broad spectrum antimicrobial that has activity against Gram positive and Gram negative bacteria as well as anaerobic organisms including resistant to Extended spectrum beta-lactamases (ESBL) and Amp C- β lactamases (Zhanel *et al.*, 2005). Carbapenem bind to penicillin-binding proteins (PBPs), prevent cross-linking of peptidoglycan and disturbing the growth and structural integrity of bacterial cell walls (Nicolau, 2008).

Imipinem is the first carbapenem derivatives of thienamycin and is slightly more active against Gram positive organisms (Nicolau, 2008). However imipinem is subjected to hydrolysed by dehydropeptidase I (DHP-I), found in the human renal brush border (Graham *et al.*, 1987). It need to be co- administer with DHP-I inhibitor which is cilastatin (Norrby *et al.*, 1983). This co-administration with cilastatin is important to prevent imipinem from destruction by dehydropeptidase. Imipinem has increased incidence of seizures (Zhanel *et al.*, 1998).

Further modification of the structural composition of the carbapenem leads to release of meropenem, ertapenem and doripenem which is more stable and not destructed by DHP-I.

Imipinem has slightly more activity against Gram positive organism where else meropenem has slightly more activity against Gram negative organism (Nicolau, 2008). Ertapenem has limited activity for non-fermenting Gram negative bacteria where else doripenem has more activity against *Pseudomonas aeruginosa* (Nicolau, 2008). Both imipinem and meropenem has short half- life and require multiple dosing where else ertapenem possess a longer serum half-life and only needs once daily dosing (Zhanel *et al.*, 2007).

Carbapenem enter Gram negative bacteria through porins. Once in the periplasmic space, carbapenem permanently acylate Penicillin binding proteins (PBPs). Penicillin binding proteins were enzymes that catalyze the formation of peptidoglycan in the cell wall bacteria. When carbapenem binds to PBPs it inhibits peptide cross linking as well other peptidase reaction and causes weakening of the peptidoglycan thus cell lysis due to osmotic pressure (Papp-Wallace *et al*., 2011). Carbapenem have the ability to bind at multiple PBPs, renders them to become more efficacy (Hashizume *et al*., 1984)

2.8 Definition of CRE

CDC previously in 2012 CRE Toolkit defines CRE as *Enterobacteriaceae* that is non-susceptible to imipinem, meropenem or doripenem and resistant to all third generation cephalosporin. However this definition was difficult to implement due to multiple antimicrobials included in the definition and also the different criteria used for carbapenem (nonsusceptible) and cephalosporin (resistant).

In January 2015, CDC has changed the CRE definition to *Enterobacteriaceae* which is resistant to imipinem, meropenem, doripenem or ertapenem or documentation of isolate possesses carbapenemase. Pre-2015 CDC CRE definition has misclassified 13% of carbapenem nonsusceptible *Klebsiella* spp, and 21% KPC-producing *Klebsiella* spp, as non-CP-CRE (Chea *et al.*, 2015). Addition of Modified Hodge Test (MHT) to the new CDC CRE definition 2015 has reduce false positive from 55% to 12% and were helpful in areas of low CRE prevalence and when dealing with organisms that were less likely to produce carbapenemase such as *Escherichia coli* or *Enterobacter* spp (Chea *et al*., 2015).

Furthermore, some of the KPC-producing CRE strain only had resistant to ertapenem while remain susceptible to imipinem and meropenem (Arnold *et al.*, 2011). This probably due to the presence of heterogenicity among isolates giving presence of susceptible in vitro and additional influences such as reduced outer membrane permeability is needed for KPC-producing CRE to possess full carbapenem resistant (Woodford *et al.*, 2004).

OXA-48 type carbapenemase also had been documented to remain susceptible against third generation cephalosporin and might be missed using previous definition (Poirel *et al.*, 2012). Non-susceptible to cephalosporin which has been included in 2012 CRE definition, does not improve the ability to differentiate between CP-CRE and non-CP-CRE.

Acceptable methods for carbapenemase detection by CDC are polymerase chain reaction (PCR), MHT, Carba-NP test or metallo-β-lactamase testing. Addition of ertapenem in new guidelines for CDC CRE-definition November 2015 increases the detection of CP-CRE and non-CP- CRE strain (CDC, 2015). Ertapenem was the most sensitive indicator for detecting KPC-producing strain compare to meropenem or imipinem (Anderson *et al.*, 2007).

2.9 Epidemiology of CRE

It is important to acknowledge CRE as one of the worrisome organisms that has spread throughout the world. Prevalence rate of CRE in Asia was still low with resistance rate of 0.8% for imipinem and 1.0% for meropenem were seen in year of 2001-2012 (Xu *et al.*, 2015). However, this could be due to only 19 out of 49 Asia countries provide epidemiology data on CRE and an incline trend of CRE rate were seen during 2009 (1.2%) and 2012 (1.3%) (Xu *et al.*, 2015). Based on National Alert Organism Surveillance 2015, CRE incidence rate in Malaysia was 0.05 of total isolates per 100 admissions. This was higher compared to 2014 whereby the incidence rate was 0.03 of total isolates per 100 admissions.

Klebsiella spp (39.26%) was the most common pathogens of CRE in Asia followed by *Escherichia coli* (21.97%), *Serratia* spp (19.8%), *Enterobacter* (12.97%), *Proteus* (3.95%) and *Citrobacter* (2.0%). *Klebsiella pneumoniae* was the most common pathogen causing CRE and this was supported by various study done in Singapore (Ling *et al.*, 2015), Taiwan (Tang *et al.*, 2016), and US (Guh *et al.*, 2015). Different pathogens were seen in Korea whereby the most common organisms seen were *Enterobacter aerogenes* (31.7%) followed by *Klebsiella pneumoniae* (19.5%) (Lee *et al.*, 2016a). Similarly in Greek, *Enterobacter* spp (49.0%) followed by *Klebsiella pneumoniae* (44.0%) was the most common CRE isolated (Miller and Johnson, 2016).

Carbapenem resistant *Enterobacteriaceae* were mostly detected in hospital settings but *Enterobacteriaceae* were common causes of community associated infections leading to probability of dissemination of CRE to community. Although CRE were commonly acquired in healthcare settings (70.5%) but community acquired CRE were seen in 29.5% of cases in Taiwan (Tang *et al.*, 2016). The clinical presentation of CRE from community was different from CRE acquired in healthcare settings whereby the community-acquired CRE were commonly among elderly female with urinary tract infections compared to healthcare-acquired CRE which were associated with intraabdominal infection, intrabdominal surgery and presence of indwelling devices (Tang *et al.*, 2016). There was also reported case in Ohio, US whereby a pregnant woman with community-acquired pyelonephritis whom urine culture revealed KPC-producing *Klebsiella pneumoniae* were successfully treated with combination of meropenem with fosfomycin and fosfomycin with cefepime (Khatri *et al.*, 2015). Community- acquired NDM-1 were also been reported previously in elderly lady with cystitis living in France (Nordmann *et al.*, 2012a).

Besides that, the presence of carbapenemase resistant gene in the mobile genetic elements such as plasmid and transposon renders the ability of these resistant gene to cross between inter/intra species (Stokes and Gillings, 2011). It also confer resistant to other antimicrobials leading to pan-resistant CRE (Zowawi *et al.*, 2015). There would be limited choice of antimicrobials and leading to higher mortality rate (40-50%) in invasive CRE infections (Patel *et al.*, 2008).

2.10 Risk Factors for CRE

The risk factors for CRE had been described previously in multiple studies. Exposure to health care and antimicrobials were among the most prominent risks for CRE (Schwaber *et al.*, 2008; Hussein *et al.*, 2009b). Exposure to antibiotics within 3 months was one of the independent risk factors for CRE (Marchaim *et al.*, 2012). Use of several classes of antimicrobials has been associated with CRKP carriage or infection which include carbapenem (Patel *et al.*, 2008; Hussein *et al.*, 2009b), cephalosporins (Patel *et al.*, 2008), fluoroquinolones (Schwaber *et al.*, 2008), glycopeptides (Jiao *et al.*, 2015; Ling *et al.*, 2015), β-lactam plus β-lactam inhibitor and metronidazole (Swaminathan *et al.*, 2013). Previous invasive procedure, diabetes mellitus, tracheostomy, solid tumor, urinary catheter insertion and antipseudomonal penicillin were identified as the risk factors for developing CRE infection in previously CRE colonizer patients (Borer *et al.*, 2012).

ICU admission, prior surgical procedure and presence of renal diseases has been among the risk factors for CRE colonization/infection (Kofteridis *et al.*, 2014). ICU admission and acute kidney injury were also risk factors for CRE acquisition in Korea (Lee *et al.*, 2016b). CRE infection/ colonization were also associated with mechanical ventilation, pulmonary diseases, days of antibiotic therapy and mean daily CRE colonization pressure in which patient were exposed (Swaminathan *et al.*, 2013). The same study also revealed that odds of acquiring CRE increased by 4% per day of antibiotic therapy.

2.11 Mechanism of resistant in CRE

Carbapenem resistant *Enterobacteriaceae* occurs from these two main mechanisms which are presence of carbapenemase genes that encodes for enzymes capable of degrading carbapenem, or overexpression of β-lactamases (AMP-C β-lactamases or ESBL) together with qualitative or/and quantitative deficiency of porin expression (Nordmann *et al.*, 2012b). Porin are proteins which form as hydrophilic channels that allow uptake of essential nutrients for bacterial survival and also antibiotics. The type of porin that usually involve in antibiotic uptakes into the periplasmic space were OmpF and OmpC (Pagès *et al.*, 2008). Any change in the type of porin presence in the outer membrane, loss of porin or modified porin can results in resistance to antibiotics. This type of carbapenem resistance usually associated with low level resistance of carbapenem.

Carbapenemase production is usually associated with high level resistance of carbapenem. There are three Ambler molecular classes of β-lactamases involved in CP-CRE which are Ambler Class A, Class B and Class D. Class C β-lactamase are mainly involved with non-CP-CRE. They are organized based on amino acid homology in the Ambler molecular classification. They are different in terms of active site of hydrolysing beta lactams and different resistance genes involved between classes. Class A, C and D need a serine residue in the active site in order to hydrolyse the β-lactams where else Class B enzymes required presence of zinc for the activity. These carbapenemase resistant genes resides in the mobile genetic elements such as transposons, integrons or plasmid causing potential spread to other isolates and from person to another person.

2.11.1 Class A beta-lactamases

Class A beta-lactamases with carbapenemase activity are encoded either on chromosomes or plasmids. Chromosomally-encoded enzymes are SME (Serratia marcescens enzyme), NMC

(non-metalloenzyme carbapenemase) and IMI (imipinem-hydrolyzing) beta-lactamases. These SME, NMC and IMI hydrolyse carbapenem, cephaloridine (1st generation cephalosporin), aztreonam and penicillin but inefficiently hydrolyse the 3rd generation cephalosporin (Queenan and Bush, 2007). SME has been found in few numbers of *Serratia marcescens* in US (Queenan *et al.*, 2000). Where else IMI and NMC were found from *Enterobacter cloacae* in US and France (Pottumarthy, 2003; Naas *et al.*, 2012).

KPC (*Klebsiella pneumoniae* carbapenemase) and GES (Guaiana-extended spectrum) were the two plasmid encoded Class A beta lactamases enzymes. GES were found in class 1 integrons where else KPC were found in transposons elements in plasmid (Walther-Rasmussen and Høiby, 2007). KPC were the most common carbapenemase enzymes seen in most part of US, although were commonly seen in *Klebsiella pneumoniae*, other *Enterobacteriaceae* and non-*Enterobacteriaceae* like *Pseudomonas aeruginosa* and *Acinetobacter* spp were also have been reported harbouring KPC (Arnold *et al.*, 2011).

2.11.2 Class B beta-lactamases

Class B beta-lactamases is also known as metallo-beta-lactamases (MBLs). They depend on zinc at their active site for hydrolysing beta-lactam drugs. They were inhibited by Ethylene Diamine Tetra Acetic Acid (EDTA) which was an ion chelator but were not inhibited by beta-lactamase inhibitor such as tazobactam, clavulanic acid and sulbactam (Juan *et al.*, 2008). They hydrolyze all beta-lactam drugs except for monobactams. The most common enzymes included in this class B beta-lactamases were New Delhi Metallo-beta-lactamase (NDM), Imipinemase Metallo-beta-lactamase (IMP), Verona integron-encoded Metallo-beta-lactamase (VIM), Sao Paolo Metallo-beta-lactamase (SPM-1), German-imipinemase (GIM) and Seoul-imipinemase (SIM-1). VIM was first detected in northern Italy in 1997 (Lauretti *et*

al., 1999). Later on VIM was isolated in Turkey (Yildirim *et al.*, 2013) and Russia (Shevchenko *et al.*, 2012). SPM-1 were seen in *Pseudomonas aeruginosa* strain in Sao Paolo, Brazil (Carvalho *et al.*, 2006). GIM-1 was isolated from *Pseudomonas aeruginosa* strain (Castanheira *et al.*, 2004). GIM-1 has also been isolated from *Enterobacter cloacae* strain in German (Hamprecht *et al.*, 2012). SIM-1 was isolated in Korea from *Pseudomonas* sp and *Acinetobacter* sp isolates.

The first MBLs detected was IMP-1 from *Pseudomonas aeruginosa* in Japan in 1991 (Watanabe *et al.*, 1991). There after IMP has been spread to many other countries including Italy (Riccio *et al.*, 2000), US (Limbago *et al.*, 2011), Australia (Sidjabat *et al.*, 2015), China (Li *et al.*, 2012) and Singapore (Koh *et al.*, 2001) . The first IMP-4 was detected in *Acinetobacter* sp strain in Hong Kong (Chu *et al.*, 2001). IMP-4 also has been detected in *Citrobacter youngae* strain in China (Hawkey *et al.*, 2001). In Malaysia, the first *bla*-$_{IMP7}$ was detected in *Pseudomonas aeruginosa* from a peritoneal fluid taken from a child with peritonitis secondary to peritoneal dialysis (Ho *et al.*, 2002). Subsequently *bla*-$_{IMP4}$ and *bla*-$_{VIM2}$ was detected in imipinem resistant *Pseudomonas aeruginosa* strain in Malaysia (Khosravi *et al.*, 2010).

Although initially *bla*-$_{IMP}$ was seen mainly in *Pseudomonas aeruginosa* but it has been reported in *Enterobacteriaceae* (Leung *et al.*, 2013) and *Acinetobacter baumanii* (Chu *et al.*, 2001). Six isolates (1.87%) of Carbapenem-Resistant *Klebsiella pneumonia* with *bla*-$_{IMP4}$ gene were detected in Malaysia (Hamzan *et al.*, 2015). It indicates the spread of this resistant gene to other species. IMP has been found as gene cassette in class 1 integrons (Arakawa *et al.*, 1995). It has been occasionally found in class 3 integrons (Shibata *et al.*, 2003). Integrons were the genetic structures that have the ability to incorporate the gene cassette encoding the resistance gene using the site-specific recombination mechanism. IMP-4 share 90-99% amino acid identity with IMP-1 (Nordmann and Poirel, 2002).

The *bla*-$_{NDM-1}$ was found in *Klebsiella pneumoniae* isolates from a Swedish who has been previously hospitalized in New Delhi, India (Yong *et al.*, 2009). Since then, NDM-1 has spread to many continents except South America and Antarctica (Johnson and Woodford, 2013). Later on NDM-1 has been discovered in seepage sample and drinking water sample in New Delhi (Walsh *et al.*, 2011). It also has been found in seepage water from river in Vietnam (Isozumi *et al.*, 2012). NDM was unique compared to other MBLs and only 32.4% identical to VIM-1/VIM-2 which has the most similarity with NDM compared to other MBLs (Yong *et al.*, 2009). Besides that, NDM was not found as gene cassette in class 1 integrons but was seen in the plasmid (Yong *et al.*, 2009). Majority of other MBLs such as IMP, VIM, GIM and SIM were found in class 1 integrons. As for biochemical characteristics of NDM, it binds stronger to zinc ions compared to other MBLs and thus more susceptible to EDTA (Li *et al.*, 2013). EDTA inactivates the zinc containing active site of the MBLs but it is toxic for clinical use. *bla*-$_{NDM-1}$ (64%) was the most common carbapenemase gene seen in Singapore (Balm *et al.*, 2013).

2.11.3 Class D beta-lactamase

Class D beta-lactamases also known as OXA-type-enzymes or oxacilinases can be divided into several subgroups which include OXA-48, OXA-51, OXA-58, OXA-143, OXA-24/40, OXA-23. Majority of them especially OXA-51, were seen on *Acinetobacter* spp, particularly *Acinetobacter baumanii* (Poirel et al., 2010; A Evans et al., 2013) except for OXA-48 where it has been isolated in *Enterobacteriaceae* (Glupczynski *et al.*, 2012). This class D beta-lactamase OXA-48 hydrolyse penicillin and carbapenem but spares the expanded-spectrum cephalosporin (Poirel *et al.*, 2010). This OXA-48 strain *Klebsiella pneumoniae* was initially identified in Turkey (Poirel *et al.*, 2004) and then further spread to France (Levast *et al.*,

2011), Greece (Voulgari *et al.*, 2013), Netherlands (Potron *et al.*, 2011a) and South Africa (Brink *et al.*, 2013). OXA-48 was still the most common carbapenemase enzymes in Turkey (Baran and Aksu, 2016). OXA-181 are different with OXA-48 by four amino acids substitution (Potron *et al.*, 2011b). It was the second most common carbapenemase gene in Singapore (Balm *et al.*, 2013).

2.12 Clinical features of CRE

CRE can cause either infection or colonization. There were several types of infections seen in CRE positive patient such as blood stream infection, ventilator associated pneumonia, urinary tract infection, intraabdominal infection (Gopi Patel *et al.*, 2008; Nguyen *et al.*, 2010; Marchaim *et al.*, 2011; Tuon *et al.*, 2016). There were 59% cases of symptomatic infection and 41% cases of asymptomatic colonization in 16 hospitals at South eastern United States (Thaden *et al.*, 2014). Eight point eight percent (8.8%) of carriers later had a positive CRE clinical specimen and exposure to fluoroquinolone and metronidazole were to be associated with subsequent CRE clinical infection (Schechner *et al.*, 2013). Thirty nine percent (39%) of CRE rectal carriage was still positive up to 1 year (Zimmerman *et al.*, 2013).

Although Gram negative organisms rarely cause skin and soft tissue infections but they become more prevalent nowadays (Lee *et al.*, 2005). There was reported cases of complicated skin and soft tissue infections caused by ESBL-producing *Klebsiella pneumoniae* (Perez, 2007). CP-CRE caused by OXA-48 producing-*Klebsiella pneumoniae* has been reported to cause outbreak of surgical site infection in Spain (Paño-Pardo *et al.*, 2012).

2.13 Laboratory diagnosis of CRE

Revised interpretative criteria for carbapenems were first published in CLSI M-100-S20-U in June 2010. Previous Clinical and Laboratory Standards Institute (CLSI) guideline before June 2010 has higher carbapenem breakpoints for imipinem, meropenem and ertapenem and has no breakpoints for doripenem. Where else CLSI guideline after June 2010 using a lower breakpoints for carbepenem and also include a breakpoint for doripenem. Details in the carbapenem break point were presented in the Table 2.1.

Other phenotypic method for detection of CRE producing MBL include EDTA-double disc synergy test which look for synergistic inhibition zone and EDTA-combined disc test which look into difference in the zone of inhibition. Double disc synergy test shows highest sensitivity (100%) and specificity (96%) for detecting MBL in *bla*-$_{VIM}$ isolates that were tested and confirmed with molecular method (Galani *et al.*, 2008). Where else another studies shows that combined disc test shows the highest sensitivity for detecting MBL in Egypt (94.6%) (El-Ghazzawy *et al.*, 2016) and Nepal (100%) (Bora *et al.*, 2014). However this combined disc test and double disc synergy test cannot be used in detecting class A and Class D carbapenemase.

Carbapenemase detection is not necessarily done for diagnosis of CRE if the laboratories are using the newer breakpoint for carbapenemase MIC. However carbapenemase detection is important for infection control particularly when outbreak occurs or in terms of for understanding the epidemiology of CRE within geographical area. Certain more aggressive infection controls such as screening contacts and patient and staff cohorting can be reserved for those with CP-CRE as this CP-CRE were believed to have a bigger potential of spread (CDC, 2016).

Modified Hodge Test (MHT) was found to have high sensitivity for detecting class A and class D carbapenemase but lower sensitivity (50%) and specificity (38.9%) for NDM (Girlich *et al.*, 2012). Furthermore this test also subjected to false positive seen in cases of non-carbapenemase CRE (Carvalhaes *et al.*, 2009). 31%-35% of non-carbapenemase-producing *Enterobacter* spp has been misclassified as carbapenemase producer by MHT (Chea *et al*., 2015). However, MHT is still used in most laboratories as one of the method for confirmation of CP-CRE as it is a simple procedure and financially can be done in many limited resources laboratories.

Other newer method for detecting carbapenemase include Carba NP test which used microtube method that detect the change in the pH colour from red to yellow in the presence of carbapenemase and produce rapid results less than 2 hours. It can be used not only in *Enterobacteriacea* but also can be used to detect carbapenemases enzymes in *Pseudomonas aeruginosa* and *Acinetobacter* species. It has 100% specificity and positive predictive value but a lower sensitivity (72.5%) and negative predictive value (69.2%) and false negative were seen in OXA-48-like producers (Tijet *et al.*, 2013). However, a revised method of Carba NP test showed 100% sensitivity and specificity for detection of all carbapenemase type of *Enterobacteriacea* and *Pseudomonas aeruginosa* isolates including OXA-48-like producers in comparison with PCR method (Dortet *et al.*, 2014).

Molecular method provides the most comprehensive review of the carbapenemase production of CRE. Specific genes involved can be determined which was important for epidemiological purpose and future plan of research. An example was the newer antibiotics ceftazidime-avibactam which provide potential use of alternative treatment for KPC- producers but not for MBL (Sader *et al.*, 2014). Therefore it is important of genotyping within geographical area. PCR method also can detect low level resistance CRE which were difficult to be detected in phenotypic test.